INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification[4] : C12P 21/00, C12N 1/20 C07K 13/00, C07H 15/12 A01H 1/04	**A1**	(11) International Publication Number: WO 88/ 08034 (43) International Publication Date: 20 October 1988 (20.10.88)

(21) International Application Number: PCT/US88/01132 (22) International Filing Date: 7 April 1988 (07.04.88) (31) Priority Application Number: 039,542 (32) Priority Date: 16 April 1987 (16.04.87) (33) Priority Country: US (71) Applicant: ECOGEN, INCORPORATED [US/US]; 2005 Cabot Boulevard West, Langhorne, PA 19047 (US). (72) Inventor: DONOVAN, William, Preston ; 650 Bayberry Lane, Yardley, PA 19067 (US). (74) Agent: LAWRENCE, Stanton, T., III; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).	(81) Designated States: AT (European patent), AU, BB, BE (European patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK, FI, FR (European patent), GA (OAPI patent), GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent). **Published** *With international search report.* *Before the expiration of the time limit for amending the* *claims and to be republished in the event of the receipt* *of amendments.*

(54) Title: BACILLUS THURINGIENSIS P-2 TOXIN GENE, PROTEIN AND RELATED INSECTICIDE COMPOSITIONS

(57) Abstract

This invention relates to a crystalline protein toxin useful as a biological insecticide which is known as P-2 toxin, or P-2 delta-endotoxin and which is produced by *Bacillus thuringiensis.* More specifically, this invention relates to the cloning and expression in various microorganisms of the gene coding for the P-2 delta-endotoxin, and related novel insecticide compositions incorporating P-2 toxin.

<u>Bacillus thuringiensis</u> P-2 TOXIN
GENE, PROTEIN AND RELATED INSECTICIDE COMPOSITIONS

1.0   INTRODUCTION

5

This invention relates to a crystalline protein
which is useful as a biological insecticide and is
known as P-2 toxin, or P-2 delta-endotoxin. It is
naturally produced by certain strains of <u>Bacillus</u>
10 <u>thuringiensis</u>. More specifically, this invention relates
to the cloning and expression in various microorganisms
of the gene coding for the P-2 delta-endotoxin, and
related novel insecticide compositions incorporating the
P-2 toxin itself and microorganisms transformed with the
15 P-2 gene.

2.0   BACKGROUND OF THE INVENTION

2.1   COMMERCIAL PESTICIDES: GENERAL CONSIDERATIONS

20

Each year, significant portions of the world's
commercially important agricultural crops are lost to
insects and other pest infestation. The damage wrought
by these pests extends to all areas of commercially
important plants including foods, textiles, and various
25 domestic plants, and the economic damage runs well into
the millions of dollars. Thus, protection of crops from
such infestations is of paramount concern.

Broad spectrum pesticides are most commonly
30 used for crop protection, but indiscriminate use of these
agents can lead to disruption of the plant's natural
defensive agents. Furthermore, because of their broad
spectrum of activity, the chemical pesticides may destroy
35 non-target organisms such as beneficial insects and

parasites of destructive pests. These are also
frequently toxic to animals and humans and, thus, pose
environmental hazards when applied.

5   Additionally, insects and other organisms have
frequently developed resistance to these pesticides when
repeatedly exposed to them. In addition to reducing the
utility of the pesticide, resistant strains of minor
pests may become major infestation problems due to the
10   reduction of beneficial parasitic organisms.

             This is a major problem encountered in using
broad spectrum pesticides. What is needed is a
biodegradable pesticide that combines a narrower spectrum
of activity with the ability to maintain its activity
15   over an extended period of time, i.e., to which
resistance develops much more slowly, or not at all.
Biopesticides appear to be useful in this regard.

2.2.   BIOLOGICAL PESTICIDES

             Biopesticides, also called biorationals, make
use of naturally occurring pathogens to control insects,
fungal, and weed infestations of agricultural crops.
Such substances may comprise a bacterium which produce a
25   substance toxic to the infesting agent (such as a toxin),
with or without a bacterial growth medium. Such bacteria
can be applied directly to the plants by standard methods
of application and will typically persist on the crops
for an extended period of time, decreasing the need for
repeat applications.

             The use of biological methods of pest control
was first suggested in 1895 when a fungal disease was
discovered in silkworms. It was not until 1940, however,
35

when spores of the milky disease bacterium <u>Bacillus</u>
<u>popilliae</u> were used to control the Japanese beetle, that
successful biological pest control was first achieved.
In the late 1960's, the discovery of a new strain of
bacterium that secreted a toxin fatal to caterpillars set
the stage for commercial biopesticides The bacterium,
named <u>Bacillus</u> <u>thuringiensis</u> (hereinafter referred to
alternatively as "B.t.") is currently the most widely
used biopesticide.

2.3   <u>BACILLUS THURINGIENSIS AND DELTA-ENDOTOXINS</u>

<u>Bacillus</u> <u>thuringiensis</u> is a widely distributed,
rod shaped, aerobic and spore forming microorganism.
During its sporulation cycle B.t. forms proteins known as
protoxins or delta-endotoxins. These protoxins are
deposited in B.t. as parasporal, crystalline inclusions
or as part of the spore coat. The patogenicity of B.t.
to a variety of sensitive insects, such as those in the
Order Lepidoptera and Diptera, is essentially due to this
parasporal crystal, which may represent over 20% of the
dry weight of the B.t. cell at the time of sporulation.

The parasporal crystal is active in the insect
only after ingestion. For instance, after ingestion by a
lepidopteran insect, the alkaline pH and proteolytic
enzymes in the mid-gut activate the crystal allowing the
release of the toxic components. These toxic components
poison the mid-gut cells causing the insect to cease
feeding and, eventually, to die. In fact, B.t. has
proven to be an effective and environmentally safe
insecticide in dealing with lepidopteran pests.

It has been reported that different strains of
B.t. produce serologically different parasporal crystals.

However, one of the predominant crystal forms produced by
many of the B.t. strains is a form known as P-1. P-1 has
a molecular weight of about 130,000-dalton and it is also
thought to be a major component of the spore coat. The
genes for the parasporal crystal P-1 and those of most of
the other protein crystals, have been discovered to
reside on any one of a large number of diferent plasmids
of varying size in B.t.

2.4 DELTA-ENDOTOXIN GENE CLONING

Since B.t. toxin genes typically reside on
plasmids and their products have proven to be effective
insecticides which are readily isolated when in
crystaline form or when associated with spore formation,
they have been the subject of a great deal of scientific
study, particularly with regard to gene isolation and
cloning procedures.

The gene which codes for P-1 has been isolated
from B.t. subspecies kurstaki strain HD-1-Dipel, and
cloned and expressed in E. coli [Schnepf et al., U.S.
Patent 4,467,036]. The protein product, P-1, was
determined to be toxic to a lepidopteran insect (tobacco
hornworm larvae). The nucleotide sequence of the
promoter region and part of the coding region of the
crystal protein gene for P-1 have also been determined
[H.P. Wong et al., The Journal of Biological Chemistry,
Vol. 258, No. 3, pp.1960-1967 (1983)]. The entire
nucleotide sequence of this gene has also been determined
and the delta-endotoxin protein itself has been expressed
in a transformed E. coli strain. [M.J. Adang et al.,
Gene, Vol, 36, pp.298-300 (1985) and PCT application
PCT/US85/01665, for: B.t. Crystal Protein Gene Toxin
Segment, (1985)].

-5-

The genes for other delta-endotoxin forms have
also been cloned and expressed in E. coli. Recombinant
plasmids containing a mosquitocidal delta-endotoxin gene
from B.t. var. israelensis was inserted into an E. coli
5   vector. A 26,000-dalton polypeptide was synthesized by
E. coli transformed with this vector. This polypeptide
was shown to be lethal to insects in the order diptera
(mosquitos). [E.S. Ward et al., FEBS Vol. 175, 2,
pp.377-382, 1984]. The nucleotide sequence of the gene
10 coding for this crystal protein was also determined along
with the resultant protein sequence [C. Waalwijk et al.,
Nucleic Acids Research, Vol.13, No. 22, pp.8207-8217,
(1985)]. Another B.t. var. israelensis gene encoding a
130 KDa crystal protein was cloned and used to transform
15 Bacillus megaterium and Bacillus subtilis. Both B.
megaterium and B. subtilis expressed crystalline
inclusions during sporulation which inclusions were
determined to be toxic to the larvae of Aedes aegypti.
[V. Sekar et al., Gene, Vol. 33, pp.151-158, (1985)].

20
Another delta-endotoxin protein crystal was
derived from B.t. subspecies sotto. The gene coding for
this crystalline protein was cloned in a vector and then
expressed in a transformed E. coli. This gene codes for
a 144,000 dalton peptide (934 amino acid residues). The
25 nucleotide sequence for the gene and the amino acid
sequence of the corresponding protein have been reported.
[Y. Shibano et al., Gene, Vol. 34, pp.243-251, (1985)].

It has also been recognized that another major
30 delta-endotoxin protein is produced by several subspecies
of B.t. [T. Yamamoto, Biochem. and Biophys. Res. Comm.
Vol. 103, No. 2, pp.414-421 (1981); T. Yamamoto et al.
Archives of Biochemistry and Biophysics, Vol. 227, No. 1,
pp.233-241 (1983)]. This delta-endotoxin has been
35

identified as P-2 and isolated from B.t. var. <u>kurstaki</u> (HD-1). This delta-endotoxin protein has a molecular of approximately 65,000 daltons and is known to be toxic to lepidoptera and diptera insects. In contrast, P-1 is

5 active only against insects of the order lepidoptera. To date, although the P-2 protein had been isolated and characterized by its activity against certain insects, the gene coding for this protein and the protein sequence itself, have remained elusive. This fact has rendered it

10 impossible to provide a means for expressing this uniquely active delta-endotoxin protein in an organism other than B.t. The availability of a cloned P-2 gene would enable the enhanced production of the P-2 protein in B.t. and also enable P-2 synthesis in a heterologous

15 organism free of other delta-endotoxins.

3.0 SUMMARY OF THE INVENTION

This invention relates to the P-2 delta-
20 endotoxin produced by <u>Bacillus</u> <u>thuringiensis</u>, the DNA sequence for the gene which codes for this protein and novel insecticides incorporating this protein and/or organisms transformed with the P-2 gene. More specifically, this invention relates to the cloning and
25 transformation of microorganisms with the gene coding for the P-2 delta-endotoxin. This invention is particularly useful in enabling the expression in organisms other than B.t. of the P-2 delta-endotoxin in quantities greater than that produced by a native P-2 producing B.t.
30 organism during sporulation. In addition, this invention is useful in permitting the transformation of a non-sporulating microorganism with the gene coding for the P-2 toxin so that this delta-endotoxin may be produced during virtually all stages of microorganism growth and,

35

thereby, not be limited to production only during a
sporulation stage.

It is an additional object of this invention to
provide a homogenous P-2 protein produced by the isolated
gene. This protein may be produced by the process of
transforming a microorganism, sporulating or non-
sporulating, such as Bacillus megaterium or E. coli or a
different strain of B.t. with the cloned P-2 gene. This
process by virtue of selection of the appropriate host
and vector would permit high yield production of the P-2
delta-endotoxin such that it is possible to derive a
substantially homogenous preparation of the P-2 toxin,
i.e. minus any contamination by other varieties of
delta-endotoxin typically produced in conjunction with or
concurrently with the P-2 toxin in its native B.t. host.
The P-2 protein and/or the transformed host may be
utilized in a variety of insecticidal composition.

It is further an object of this invention to
provide an organism, other than the native B.t. host,
transformed with the DNA coding for the P-2 delta-
endotoxin. This foreign transformed host enables the
production of the P-2 delta-endotoxin under more
desirable and/or selective culturing conditions.

It is another object of this invention to
provide a DNA probe useful for detecting the presence of
the P-2 gene in the various Bacillus thuringiensis
strains. This DNA probe also enables the screening of
various strains of B.t. for the possible presence of
related genes coding for proteins sharing a common
homology with the P-2 protein and the isolation of these
related genes. All of the above embodiments of this

invention will be described in greater detail in the
description of the invention which follows.

4.0 BRIEF DESCRIPTION OF THE FIGURES

5

FIGURE 1 is a restriction map of the
recombinant plasmids pEG 201 and pEG 204 that contain the
cloned P-2 gene. The location and direction of
transcription of the P-2 gene are indicated by the large
10   arrow.

FIGURE 2 shows the DNA nucleotide sequence of
the P-2 gene and also the amino acid sequence of the P-2
protein coded for by the DNA nucleotide sequence.

15

FIGURE 3 is comprised of 3A and 3B. 3A is a
photograph of an ethidium bromide stained Eckhardt gel.
The native plasmids that are present in various strains
of B.t are visible illustrating that most strains of B.t.
contain several native plasmids. 3B is a photograph of
.0   an autoradiogram that was made by hybridizing the
radioactively labeled cloned P2 gene with the plasmids
shown in 3A. 3B illustrates that the cloned P2 gene
hybridized exclusively to a plasmid of 110 MDa in three
strains of B.t. that were known to produce P2 protein
25   (HD1-1, HD263-1 and HD278). The cloned P2 gene also
hybridized to a DNA band of 30 MDa (3B).

FIGURE 4 is comprised of 4A and 4B. 4A is a
photograph of an ethidium bromide stained agarose gel
30   that contains HindIII digested DNA from strains HD1-1,
HD263-1, HD267 and HD278. 4A shows that total B.t. DNA
that had been digested with HindIII could be resolved
into hundreds of different sized fragments. 4B is a
photograph of an autoradiogram that was made by
35

hybridizing the radioactively labeled cloned P2 gene with the HindIII fragments shown in 4A. 4C is a photograph of an autoradiogram that was made after re-washing the nitrocellulose filter of 4B at 80°C.

5

Figure 5 is a photograph of an SDS/polyacrylamide gel which shows that a recombinant host strain of Bacillus megaterium harboring the cloned P-2 gene synthesizes large quantities of a protein having a similar size as that of authentic P-2 protein.

10

Figure 6 shows the region of homology between the amino acid sequence of P-1 toxin and P-2 toxin.

15

5.0   DESCRIPTION OF THE INVENTION

Generally stated, the present invention provides for a cloned gene coding for Bacillus thuringiensis P-2 delta-endotoxin or toxin and comprising the DNA nucleotide sequence shown in FIG. 2. This gene (which comprises double stranded DNA wherein the nucleotide strands have a complementary base sequence to each other) codes for a protein (or as also used herein equivalently, polypeptide) having the amino acid sequence of the P-2 toxin which amino acid sequence is shown in FIG. 2. The P-2 toxin encoded by the cloned gene has insecticidal activity against lepidoptera and diptera insects.

20

25

Methods of producing the P-2 protein are also provided by this invention. In this method of production the P-2 delta-endotoxin gene is inserted into a cloning vector or plasmid which plasmid is then utilized to transform a selected microorganism.

30

35

The cloning vectors, as described herein, are
generally known in the art and are commercially
available. The choice of a particular plasmid is within
the skill of the art and would be a matter of personal
choice. Plasmids suitable for use in this invention are,
for instance, pBR322, plasmids derived from B.t., and
plasmids derived from Bacillus microorganisms and,
preferrably, are those such as, preferrably, Bacillus
megaterium. Microorganisms suitable for use with this
invention are both sporulating and non-sporulating
microorganism such as E. coli, B.t., and Bacillus
megaterium. The microorganisms utilized are also known
in the art and are generally available. The choice of
any particular microorganism for use in the practice of
this invention is also a matter of individual preference.
In a preferred embodiment of this invention the
microorganism would comprise Bacillus megaterium.

Generally stated, the P-2 toxin protein can be
produced by a transformed organism and later purified
into a homogenous preparation having an amino acid
sequence as shown in FIG. 2. More specifically, this
protein may be produced by transforming a microorganism
with the P-2 gene, growing the transformed microorganism
so that the protein coded for by the P-2 gene is
expressed in the microorganism and by extracting the
protein from the organism with standard protein
purification techniques. It is also within the scope of
this invention that the protein not be separated from the
transformed microorganism but that this organism,
including the expressed P-2 protein, be utilized as or in
an insecticidal composition.

This invention also provides for a novel
insecticide for use against lepidoptera and diptera

comprising a mixture of B.t. P-2 toxin and a suitable
carrier. The P-2 toxin may be contained in the organism
or as part of spores, or be a homogenous protein
preparation or in a mixture of spores with cultured
transformed organisms. The P-2 toxin may also be
contained in a non-sporulating microorganism or a
sporulating microorganism such as <u>Bacillus megaterium</u> or
B.t. A suitable carrier may be any one of a number of
solids or liquids known to those of skill in the art.

          This invention also comprises the recombinant
vectors or plasmids including the P-2 gene and the
particular microorganisms which have been transformed
with this gene. In addition, this invention also
provides for oligonucleotide probes for the gene coding
for the P-2 delta-endotoxin. All of these aspects of the
inventions are described in detail below and illustrated
in the following examples.

5.1 <u>RECOMBINANT DNA TECHNOLOGY AND GENE EXPRESSION</u>

          Generally stated, recombinant DNA technology
involves insertion of specific DNA sequences into a DNA
vehicle (plasmid or vector) to form a chimeric DNA
molecule which is capable of replication in a host cell.
The inserted DNA sequence is typically foreign to the
recipient host, i.e, the inserted DNA sequence and the
DNA vector are derived from organisms which do not
exchange genetic information in nature, or the inserted
DNA sequence may be wholly or partially synthetically
made. In recent years several general methods have been
developed which enable construction of recombinant DNA
molecules, For example, U.S. Pat. No. 4,237,224 to Cohen
and Boyer describes production of such recombinant
plasmids using restriction enzymes and methods known as

ligation. These recombinant plasmids are then introduced
and replicated in unicellular organisms by means of
transformation. Because of the general applicability of
the techniques described therein, U.S. Pat. No. 4,237,224
is hereby incorporated by reference into the present
specification.

Regardless of the method used for construction,
the recombinant DNA molecule must be compatible with the
host cell, i.e., capable of autonomous replication in the
host cell. The recombinant DNA molecule should also have
a marker function which allows the selection of host
cells so transformed by the recombinant DNA molecule. In
addition, if all of the proper replication, transcription
and translation signals are correctly arranged on the
chimeric DNA molecule, the foreign gene will be expressed
in the transformed cells and their progeny.

These different genetic signals and processing
events control many levels of gene expression, i.e., DNA
transcription and messenger RNA translation. Trans-
cription of DNA is dependent upon the presence of a
promoter which is a DNA sequence that directs the binding
of RNA polymerase and thereby promotes transcription.

Translation of messenger RNA (mRNA) in
procaryotes depends upon the presence of the proper
procaryotic signals. Efficient translation of mRNA in
procaryotes, such as B.t., requires a ribosome binding
site called the Shine Dalgarno (SD) sequence on the mRNA.
This sequence is a short nucleotide sequence of mRNA that
is located before the start codon (AUG) which encodes the
amino-terminal methionine of the protein. The SD
sequences are complementary to the 3'-end of the 16S RNA
(ribosomal RNA) and probably promote binding of mRNA to

robosomes by duplexing with the mRNA to allow correct
positioning of the ribosome (Roberts and Lauer, 1979,
Methods in Enzymology, 68:473).

5   One method widely employed for the cloning of a
particular gene is to prepare a "library" of recombinant
plasmids. Each recombinant plasmid is comprised of a
plasmid vector, which usually confers antibiotic
resistance to cells that harbor it, plus a fragment of
10   DNA from the donor organism, an organism that contains
the gene. The plasmid library is commonly prepared by
digestion of both the plasmid vector and total DNA from
the donor organism with a restriction enzyme,
inactivation of the enzyme and ligation of the DNA
15   mixture. The ligated DNA is a plasmid library. The key
feature of this plasmid library is that it contains many
different recombinant plasmids. It is highly likely that
at least one of the recombinant plasmids in the library
will contain a fragment of DNA from the donor organism on
which the gene of interest resides. The plasmid library
20   is transformed into the cells of a host organism that
does not contain the gene. The host cells are spread on
a selective solid medium, usually one containing an
antibiotic, that allows only transformed cells, those
containing recombinant plasmids, to grow into colonies.
25   Individual transformed host colonies are tested for the
acquisition of the gene from the donor organism. In host
colonies the acquired gene is carried on the recombinant
plasmid.

30
        One of the most direct methods of testing for
the acquisition of a gene is to use a gene-specific
hybridization probe, a fragment of DNA that is homologous
to the gene. A characteristic of homologous DNA
fragments is that they will bind tightly to each other
35

during hybridization. Typically a radioactively labeled
DNA probe is used during hybridization so that binding of
the probe to the gene can be easily monitored.

5   A recent advance in molecular biology is the
use of synthetic oligonucleotides as gene-specific
probes. The basis for the use of the oligonucleotides is
that in all biological systems a particular sequence of
nucleotides encodes a precise sequence of amino acids.
10   Conversely if the sequence of amino acids is known for a
particular protein then the nucleotide sequence encoding
the protein can be inferred, although not precisely. In
practice, the partial amino acid sequence of a protein,
the product of the gene of interest, is determined by
15   chemical methods. Based on the protein amino acid
sequence a gene-specific oligonucleotide probe is
synthesized that may be, to varying degrees, homologous
to the gene. Exact homology cannot be guaranteed because
knowledge of the amino acid sequence of a protein does
give exact knowledge of the nucleotide sequence of the
gene encoding the protein. Nevertheless, even though the
homology between the oligonucleotide probe and the gene
may not be precise, hybridization conditions can usually
be found that will permit the oligonucleotide probe to
bind specifically to the gene.
25

          Accordingly, in isolating the P-2 gene, the P-2
protein was purified from a donor strain of B.
thuringiensis var. kurstaki, and the partial amino acid
sequence of the P-2 protein was determined. A P-2 gene-
30   specific oligonucleotide probe was synthesized based on
the amino acid sequence of the P-2 protein. The
oligonucleotide was radioactively labeled and was used in
hybridization experiments to identify transformed host

35

colonies that harbored recombinant plasmids carrying the
P-2 gene from the donor B.t. strain.

5.2   CLONING OF THE P-2 TOXIN GENE FROM
BACILLUS THURINGIENSIS STRAIN HD263-1

5

More specifically, in order to clone the P-2
toxin gene of this invention, cells of B.t. strain HD1-1,
a single colony isolate immediately derived from parent

10   strain HD-1 (U.S.D.A., Cotton Insect Research Unit,
Brownsville, Texas 78520), were grown in C2 media (1%
Glucose, 0.2% Peptone, 0.5% N Z Amine A, 0.2% Yeast
Extract, 15mM $(NH_4)_2SO_4$, 23mM KH_2PO_4, 27mM K_2HPO_4, 1mM
$MgSO_4.7H_2O$, 600uM $CaCl_2$, 17uM $ZnSO_4.7H_2O$, 17uM

15   $CuSO_4.5H_2O$, 2uM $FeSO_4.7H_2O$) at 30° C until t72 (hours)
and spores plus crystals were harvested by
centrifugation. The spore/crystal pellet was washed with
several changes of 1 M NaCl and then several changes of
deionized water. Toxin proteins were solubilized by
incubating the spore/crystal preparation in 5% B-

20   mercaptoethanol, 2% NaDodeSO4, 60 mM Tris pH 6.8, 10%
glycerol at 70 degrees C. for 7 min., and spores were
removed by centrifugation. The supernatant was
electrophoresed through polyacrylamide gels containing
NaDodeSO4 to separate proteins. The gel was stained with

25   Coomassie dye and gel slices containing the P-2 protein
were cut out with a razor blade. The homogeneous P-2
protein preparation was electroeluted from gel slices
and, after acetone precipitation, the NH_2-terminal amino
acid sequence of the P-2 protein was determined by

30   automated Edman degradation carried out on an Applied
Biosystems Gas Phase Sequenator (model 470A) and analyzed
on a DuPont Zorbax C18 column in a Hewlett-Packard HPLC
(model 1090) with a 1040 diode array detector. The amino

35

acid sequence of the NH$_2$ terminal portion of the
homogeneous P-2 protein was determined to be:

```
1    2    3    4    5    6    7    8    9   10
MET  ASN  ASN  VAL  LEU  ASN  SER  GLY  ARG  THR


11   12   13   14   15   16   17   18   19   20
THR  ILE  ASN  ASP  ALA  TYR  ASN  VAL  VAL  ALA


21   22   23   24   25   26
HIS  ASP  PRO  PHE  SER  GLY
```

5.3 OLIGONUCLEOTIDE PROBE FOR THE P-2 GENE

A 62 mer oligonucleotide probe encoding amino
acids 4 through 24 of the NH2-terminus of the P-2 protein
was synthesized on an Applied Biosystems DNA synthesizer
(model 380A). It was recognized that because of the
codon degeneracy (certain amino acids are each encoded by
several slightly different codons) the sequence of the
synthetic oligonucleotide would probably be different
from the actual NH2-terminal sequence of the P-2 gene.
However, the fact that the B.t. genome is 68% A:T and the
codon usage information for previously cloned and
sequenced B.t. genes were used in designing an
oligonucleotide probe that would have the highest
probability of matching the actual sequence of the P-2
gene. The oligonucleotide probe was designed to bind
only to the NH2-terminal coding region of the P-2 gene.
The sequence of the P-2 gene-specific oligonucleotide
probe was:

5'-GTA TTA AAT TCA GGA AGA ACA ACA ATT AAT GAT GCA TAT
AAT GTA GTA GCA CAT GAT CCA TT-3'.

-17-

        In addition to enabling the original isolation
of the P-2 gene herein, this DNA probe also comprises
another preferred embodiment of this invention. This DNA
probe permits the screeing of any B.t. strain to
5   determine whether the P-2 gene (or possibly a related
gene) is naturally present or whether a particular
transformed organism includes the P-2 gene. In this
fashion it is also possible to estimate the insectididal
activity of that strain of B.t. It is also with the
10 scope of this invention that this probe may comprise a
smaller or larger oligonucleotide. The probe may be
labeled by any number of techniques known in the art(such
as readioactively or enzymatically labeled) and as
described below.

15

20

25

30

35

5.4   CONSTRUCTION OF A PLASMID LIBRARY ENRICHED FOR THE
      P-2 GENE

        The oligonucleotide probe was used to determine

5   the size of a restriction fragment of B.t. DNA that
    contained at least the NH2-terminal coding region of the
    P-2 gene. For this determination strains HD263-1, a
    single colony isolate immediately derived from parent
    strain HD-263 (U.S.D.A., Cotton Insect Research Unit,

10 Brownsville, Texas 78520), and strain HD1-1, a single
    colony isolate immediately derived from patent strain
    HD-1 (U.S.D.A., Brownsville, Texas) were used as a source
    of DNA. Both of these strains were known to produce the
    P-2 crystal protein. B.t. strains EG2158 and HD567 which

15 do not produce P-2 crystal protein were used as negative
    controls.

        DNA was isolated from the various donor strains
    after growth of the cells to mid-log phase at 30° C in LB

20 medium. Cells were harvested by centrifugation,
    resuspended in 50mM Tris HCl pH 7.8, 10mM EDTA, 1 mg/ml
    lysozyme and incubated at 37°C for 60 min. Cells were
    lysed by adding NaDodeSO$_4$ to a final concentration of
    0.2%. Cell lysates were extracted twice with an equal

25 volume of phenol and once with an equal volume of
    chloroform/isoamyl alcohol (24/1). One tenth volume of 3
    M NaAcetate and 2 volumes of EtOH were added to the
    lysates and DNA was extracted by spooling on a glass rod.
    The spooled DNA was soaked in 66% EtOH for 5 min. and in

30 diethyl-ether for 1 min. The spooled DNA was air dried
    and resuspended in deionized water.

        Hybridization experiments were performed by
    digesting total DNA from each of the donor strains with

35 HindIII restriction enzyme, electrophoresing the digested

DNA on an agarose gel and transfering the DNA from the
agarose gel to a nitrocellulose filter by the blot
technique of Southern (J. Molec. Biol. 98:503-517, 1978).
The nitrocellulose filter was incubated at 32°C for 16

5   hrs. in a solution of 3 X SSC (1 X SSC = 0.15M NaCl/0.015
M Sodium Citrate), 0.1 % NaDodSO$_4$, 200 ug/ml heparin, 10
X Denhardt's (1 X = 0.02% Bovine Serum Albumin/0.02%
Ficoll/0.02% Polyvinyl-Pyrrolidone) containing
approximately 1 ug of the P2 gene-specific

10 oligonucleotide probe that had been radioactively labeled
with gamma-P32-ATP and T4 kinase. After hybridization
the nitrocellulose filter was washed with 3 X SSC, 0.1 %
NaDodSo$_4$ at 32°C for one hour and the filter was exposed
to X-ray film. The resulting autoradiogram showed that

15 the oligonucleotide probe specifically hybridized to two
HindIII fragments of DNA, from strain HD263-1, of
approximately 9.0 and 5.0 kb. The probe hybridized to
apparently identical 9.0 and 5.0kb HindIII fragments of
DNA from strain HD1-1. The probe failed to hybridize to

20 any DNA restriction fragments from two strains of B.t.
that did not synthesize P-2 crystal protein, EG2158 and
HD567.

It was necessary to determine which of the two
HindIII fragments, 9.0 or 5.0 kb, hybridized most

25 strongly to the oligonucleotide probe since the most
strongly hybridizing fragment would be most likely to
contain the P-2 gene. Strength of hybridization is
measured by the highest wash temperature at which the
probe remains bound to the DNA fragment on the

30 nitrocellulose filter. Accordingly the nitrocellulose ·
filter was repeatedly washed in 3 X SSC, 0.1 % NaDodSO$_4$
at progressively higher temperatures, each wash being
followed by autoradiography, until a temperature was
reached (50°C.) at which the radioactive probe no longer

35

hybridized to the 9.0 kb fragment but was seen to
hybridize exclusively to the 5.0 kb band. Therefore, it
was determined that at least the NH2-terminal coding
region of the P-2 gene resided on the 5.0 kb HindIII
5   fragment of DNA from strains HD263-1 and HD1-1.

A P-2 gene-enriched plasmid library was
constructed by digesting HD263-1 total DNA with HindIII,
electrophoresing the digested DNA on an agarose gel and
10   excising gel slices containing HindIII DNA fragments
ranging in size from approximately 4.0 to 6.0 kb.
HD263-1 HindIII fragments ranging in size from 4.0 to 6.0
kb were electroeluted from agarose gel slices, phenol
plus chloroform extracted, ethanol precipitated and
15   ligated into the HindIII site of plasmid pBR322 that had
been digested with HindIII and treated with alkaline
phosphatase. Alkaline phosphatase greatly increased the
probability that recombinant plasmids were formed
consisting of pBR322 plus a HindIII fragment of HD263-1
20   DNA. The resulting ligation mix consisted of a library
of recombinant plasmids enriched for the P-2 toxin gene
from strain HD263-1.

5.5   COLONY HYBRIDIZATION AND ISOLATION OF A 5.2 kb
      HindIII FRAGMENT CONTAINING THE P-2 GENE
25

The P-2 gene-enriched plasmid library was
transformed into an ampicillin sensitive host strain of
E. coli, HB101 (Bethesda Research Laboratories, Bethesda,
MD.), by the $CaCl_2$ procedure. E. coli strain HB101 does
30   not synthesize P-2 protein and, therefore, it would not
be expected to contain the P-2 gene. E. coli was used as
the host strain because these cells are easily
transformed with recombinant plasmids. All host cells
acquiring a recombinant plasmid would become ampicillin
35

resistant. After exposure to the recombinant plasmids
the E. coli host cells were spread onto solid medium
containing ampicillin and those cells that harbored a
recombinant plasmid were able to grow into colonies. It
5   was expected that each individual ampicillin resistant
host colony would harbor many identical copies of a
recombinant plasmid comprised of pBR322 plus a unique
HindIII fragment from the donor strain HD263-1 DNA.
However, the donor strain HindIII fragment in the
10   recombinant plasmid would differ from one colony to the
next.

        Approximately two thousand individual
ampicillin resistant colonies were blotted onto
15   nitrocellulose filters. Replicas of the colonies were
saved for later use as described below. The recombinant
plasmids contained in the colonies were bound to the
nitrocellulose filters by treating the colonies with NaOH
and NH$_4$Acetate. The resulting nitrocellulose filters
20   contained an array of recombinant plasmids each of which
was physically separated from other recombinant plasmids.
The nitrocellulose filters were hybridized at 50°C for 16
hours in a solution of 3 X SSC, 200 ug/ml heparin, 0.1%
NaDodeSO$_4$, 10 X Denhardt's and approximately 1 ug of the
25   P2 gene-specific oligonucleotide probe that had been
radioactively labeled. The filters were washed at 50°C
for one hour in 3 X SSC, 0.1% NaDodeSO$_4$ and were exposed
to x-ray film. The resulting autoradiogram showed that
the oligonucleotide probe had hybridized to recombinant
30   plasmids at four different locations on the
nitrocellulose filters.

        By aligning the autoradiogram with the colony
replicas it was possible to identify four colonies whose

35

recombinant plasmids had apparently hybridized with the
oligonucleotide probe.

5   The recombinant plasmids were extracted from
each of the four colonies. The plasmids were digested
with HindIII and electrophoresed on an agarose gel.
Three of the four plasmids consisted of pBR322 plus an
apparently identical sized 5.2 kb HindIII fragment of
HD263-1 DNA. The plasmids were transferred from the
10 agarose gel to a nitrocellulose filter by the blot
procedure of Southern. The nitrocellulose filter was
hybridized with the radioactively labeled oligonucleotide
probe and exposed to x-ray film. The resulting
autoradiogram showed that the oligonucleotide probe
15 hybridized exclusively to the 5.2 kb HindIII fragment in
each of the three recombinant plasmids. One of these
recombinant plasmids, designated pEG201, was selected for
further experimentation and evaluation. The original E.
coli colony harboring pEG 201 was designated EG 1304.

20

5.6   LOCATION OF THE P-2 GENE ON THE CLONED 5.2 KB
      HindIII FRAGMENT.

          It was likely that the cloned 5.2 kb HindIII
fragment contained at least the NH2-terminal coding
25 region of the P-2 gene. Presence of the P-2 gene on the
5.2 kb fragment was verified using DNA sequencing to
search for a region in the cloned 5.2 kb fragment that
encoded the NH2-terminus of the P-2 protein. Since it is
difficult to sequence a fragment of DNA longer than two
30 kb it was necessary to identify a smaller fragment of DNA
within the 5.2 kb fragment that would be expected to
contain the P-2 gene. Accordingly plasmid pEG201 was
digested with various restriction enzymes, digested
plasmid was electrophoresed through an agarose gel and
35

plasmid restriction fragments were blotted from the gel
to a nitrocellulose filter. Hybridization of the filter
with the radioactively labeleled oligonucleotide probe
revealed that the probe specifically hybridized to a 1.3
5   kb Sau3A Restriction fragment of DNA. Therefore, it was
expected that the 1.3 kb fragment would contain at least
the NH2-terminal coding region of the P-2 gene.

        The 1.3 kb fragment was subcloned from pEG201
10 into the DNA sequencing vectors mp18 and mp19 (Bethesda
Research Laboratories, Bethesda MD). DNA sequencing of
the 1.3 kb fragment revealed that it contained a region
of DNA that encoded the NH2-terminus of the P-2 protein.
This conclusively demonstrated that the cloned 5.2 kb
15 HindIII fragment from the donor strain HD263-1 contained
the P-2 gene. Additional DNA sequencing of the 1.3 kb
fragment showed that an AccI restriction site was located
150 nucleotides upstream from the NH2-terminal methionine
codon of the P-2 gene. The position of this AccI site
20 served as a marker. It allowed the location of the P-2
gene in the 5.2 kb fragment to be precisely determined as
described below.

        The location and direction of transcription of
the P-2 gene on the cloned 5.2 kb fragment was determined
25 by digesting the 5.2 kb fragment with AccI in combination
with various other restriction enzymes. The restriction
fragments were electrophoresed through an agarose gel and
blotted onto a nitrocellulose filter. By hybridizing the
filter with the radioactively labeled P2 gene-specific
30 oligonucleotide probe it was possible to determine the
location and orientation of various restriction fragments
on the larger 5.2 kb fragment. From this knowledge the
precise position and direction of transcription of the
P-2 gene on the 5.2 kb fragment was determined as
35

indicated by the arrow in Figure 1. Figure 1 shows a
restriction map of plasmid pEG201. The boxed areas
denote plasmid vector DNA. pBR322 vector is indicated by
an open boxed area. The horizontal line denotes cloned
5   B.t. DNA from strain HD263-1. The large arrow indicates
the coding region of the P2 gene. Plasmid pEG204 is
described below. The length of the P-2 gene was
estimated to be approximately 1.9 kb based on the
estimated size (68 kDa) of the P-2 protein.

10

5.7   DNA SEQUENCE OF THE CLONED P-2 GENE

It was estimated that all or at least most of
the P-2 gene was contained in the 2.2 kb AccI - HindIII
15   fragment within the cloned 5.2 kb fragment (Fig. 1).
Accordingly, the 2.2 kb AccI - Hind III fragment was
subcloned into the sequencing vectors mp18 and mp19 and
the complete sequence of the 2.2 kb fragment was
determined by the dideoxy procedure of Sanger (Sanger,
F., Nicklen, S. & Coulson, A.R. (1977) Proc. Natl. Acad.
20   Sci. USA 74:5463-5467). As expected the 2.2 kb fragment
contained an open reading frame (protein coding region)
that began with the NH2-terminal codons for the P-2
protein. The DNA sequence of the 2.2 kb fragment, which
includes the P-2 gene of this invention, and the deduced
25   amino acid sequence of the P-2 protein are shown in
Figure 2. Figure 2 shows the complete DNA sequence of
the 2.2. kb Accl - HindIII fragment beginning with the
first nucleotide of the Accl site and ending with the
last nucleotide of the HindIII site. The Accl site is
30   located 150 nucleotides upstream from the NH2-terminal
methionine codon of the P-2 gene. The size of the P-2
protein, as deduced from the P-2 gene sequence, was
determined to be 66,547 Daltons.

35

5.8   USE OF THE CLONED P-2 GENE AS A SPECIFIC HYBRIDIZATION PROBE.

5.8.1   IDENTIFICATION OF NATIVE B.t. PLASMIDS CONTAINING P-2 GENES.

One advantage of a cloned DNA sequence is that it can be used to identify related DNA sequences in uncharacterized samples of DNA. In the case of the P-2 gene it is now possible that the cloned gene can be used to detect the presence of a P-2 gene in a strain of B.t. Most strains of B.t. contain numerous native plasmids in addition to chromosomal DNA. For many of these strains it is not known if the P-2 gene resides on the chromosome or on one of the plasmids.

In order to determine whether the cloned P-2 gene could be used to detect the locations of a P-2 gene in a native B.t. host strain, B.t. strains HD263-1, HD1-1, HD567 and HD278 were lysed according to the procedure of Eckhardt (Eckhardt, T. (1978) Plasmid 1:584-588) and the lysates were electrophoresed through agarose gels. This procedure allowed the separation by size of all plasmids contained in a particular strain. The separated plasmids were transferred from the agarose gel to a nitrocellulose filter by the blot procedure of Southern. The nitrocellulose filter was hybridized with the radioactively labeled 2.2kb AccI - HindIII (P-2 gene) fragment. Autoradiography of the nitrocellulose filter revealed that the P-2 gene fragment hybridized exclusively to one plasmid of approximately 110 MDa in the P2-producing strains HD263-1, HD1-1 and HD278 (Figure 3). The cloned P-2 gene did not hybridize to any plasmids in the P-2-negative strain HD567. Therefore, this experiment demonstrated that the cloned P-2 gene can be

used in a direct manner to identify native plasmids
containing P-2 genes in B.t. strains. DNA hybridization
with the cloned P-2 gene allowed direct identification of
a single plasmid carrying a P-2 gene out of many such
5   plasmids existing in strains of B.t.

10

15

20

25

30

35

5.8.2   IDENTIFICATION OF DNA RESTRICTION FRAGMENTS
CONTAINING P-2 GENES.

The cloned P-2 gene from B.t. strain HD263-1
was contained on a 5.2 kb HindIII fragment of DNA. A
procedure was conducted in order to determine whether the
cloned P-2 gene could be used to identify similar DNA
restriction fragments containing P-2 genes from other
strains of B.t. Accordingly, total DNA from the test
strains HD1-1, HD278, HD567 and the original donor strain
HD263-1 were digested with HindIII restriction enzyme,
the digested DNA was electrophoresed through an agarose
gel and digested DNA was transferred from the gel to a
nitrocellulose filter. The filter was hybridized at 55°C
with the radioactively labeled 2.2 kb AccI - HindIII DNA
(P-2 gene) fragment and, after washing at 55°C, the
filter was exposed to x-ray film.

Figure 3 (3A) is a photograph of an ethidium
bromide stained Eckhardt gel. The native plasmids that
are present in various strains of B.t. are visible. 3A
illustrates that most strains of B.t. contain several
native plasmids. The numbers to the left of the figure
indicate the size in megadaltons (MDa) of the plasmids.

Figure 3 (3B) is a photograph of an
autoradiogram that was made by hybridizing the
radioactively labeled cloned P-2 gene with the plasmids
shown in 3A. 3B illustrates that the cloned P-2 gene
hybridized exclusively to a plasmid of 110 MDa in three
strains of B.t. that were known to produce P-2 protein
(HD-1, HD263-1 and HD278). The cloned P2 gene also
hybridized to a DNA band of 30 MDa (Fig. 3 (3B)). This
DNA band was DNA fragments that resulted from the
breakdown of the 110 MDa plasmid. Very large plasmids

such as the 110 MDa plasmid are often broken down into
smaller fragments during electrophoresis through
Eckhardt-type gels. Specific hybridization of the cloned
P-2 gene to the 110 MDa plasmids indicates that for
5   strains HD1-1, HD263-1 and HD278 only the 110 MDa
plasmids carry the P-2 gene. The cloned P2 gene did not
hybridize to any plasmids from a strain that did not
produce P2 protein (HD567). Overall, Figure 3 (3A and
3B) demonstrate that the cloned P-2 gene can be used as a
10   specific probe for the identification of P-2 genes on
native B.t. plasmids.

      Figure 4 (4A) is a photograph of an ethidium
bromide stained agarose gel that contains HindIII
15   digested DNA from strains HD1-1 (lane 2), HD567 (lane 3),
HD278 (lane 4) and HD263-1 (lane 5). Figure 4 (4A) shows
that total B.t. DNA that had been digested with HindIII
could be resolved into hundreds of different size
fragments. The lane marked 1 contained Lambda DNA that
20   had been digested with HindIII. This serves as a size
marker. The numbers to the left indicate the size in
kilobase (kb) of the DNA fragments.

      Figure 4 (4B) is a photograph of an
25   autoradiogram that was made by hybridizing (at 55°C) the
radioactively labeled cloned P-2 gene with the HindIII
fragments shown in 4A. Overall, Figure 4 (4A and 4B)
demonstrate that cloned P-2 gene can be used as a
specific probe for the identification of DNA restriction
fragments that contain P-2 genes.
30

      Autoradiography showed that, as expected, the
cloned P-2 gene hybridized to a 5.2 kb HindIII fragment
from strain HD263-1 (Figure 4, (4B) Lane 5).
Surprisingly, the P-2 gene also hybridized to a 9.0 kb
35

HindIII fragment from strain HD263-1 (Figure 4(4B), lane
5). The P-2 gene hybridized to 5.2 and 9.0 kb HindIII
fragments in strain HD1-1 (Figure 4(4B), lane 2), and to
HindIII fragments of 5.2 and 4.4 kb in strain HD278

5   (Figure 4(4B) lane 4). The P-2 gene failed to hybridize
to any HindIII fragments from the P-2-negative strain
HD567 (Figure 4(4B), lane 3). The nitrocellulose filter
was rewashed at 80°C and exposed to x-ray film. The
resulting autoradiogram showed that, after the higher

10   wash temperature, significantly less of the labeled P-2
gene probe was bound to the 9.0 and 4.4 kb fragments than
to the 5.2 kb fragment (Figure 4(4C), Lanes 2, 4 and 5).

       The above demonstrates several preferred uses
15   of the cloned P-2 gene. First the cloned P-2 gene from
strain HD263-1 can be used to identify DNA restriction
fragments containing P-2 genes from other B.t. strains.
For example the cloned P-2 gene hybridized to two HindIII
fragments from strain HD1-1. Second the cloned gene
permits determination of the number of P-2 genes present

20   in a particular B.t. strain. For example strain HD263-1
was found to contain two P-2 genes since the P-2 gene
probe hybridized to two HindIII restriction fragments
from strain HD263-1. It is significant that the P-2 gene
specific oligonucleotide probe also hybridized to two

25   HindIII fragments of 9.0 and 5.2 kb from strain HD263-1.

       The precise nature of the P-2 gene on the 9.0
kb fragment cannot be known until the 9.0 kb fragment is
isolated and its DNA sequence determined. This leads to

30   a third preferred use for the cloned P-2 gene. Since the
P-2 gene specifically hybridized to a 9.0 kb HindIII
fragment the cloned P-2 gene (or portions or derivatives
thereof) is therefore, an ideal probe for the isolation
of this fragment.

35

Fourth, the cloned P-2 gene permits rapid
qualitative estimation of the relatedness between P-2
genes. For example, it would be estimated that the
cloned P-2 gene is more related to the P-2 gene contained
5   on the 5.2 kb fragment of strain HD1-1 than to the P-2
genes contained on either the 9.0 or 4.4 kb HindIII
fragments. This relatedness was directly demonstrated by
the stronger hybridization at 80°C of the P-2 gene probe
to the 5.2 kb fragment than to either the 9.0 or 4.4 kb
10   fragments.

5.9 ADDITIONAL PURIFICATION OF P-2 TOXIN

The P-2 gene can be inserted in any appropriate
15   plasmid which may then be utilized to transform an
appropriate microorganism. It is clearly within the
scope of this invention that microorganisms other than
B.t. may be transformed by incorporation of the P-2 gene
i.e., generally stated, organisms from the genera
20   Bacillus, Escherichia, and Cyanobacteria. Preferred for
use with this invention are organisms Bacillus megaterium
and Escherichia coli. It is also within the scope of
this invention that different strains of B.t. may also be
transformed by the incorporation of the P-2 gene.

25
The microorganisms so transformed will
preferably produce P-2 in quantities that are far in
excess of the quantity of P-2 produced in a B.t. natural
host strain. The P-2 produced by a transformed organism
is preferably the only delta-endotoxin produced by that
30   organism. In this manner, the organism itself may be
utilized alone or as part of an insecticidal composition.
Since P-2 would preferably be the only delta-endotoxin
produced by the organism, it is a straightforward process

35

to purify the P-2 from other cellular material by methods
known in the art such as renografin density gradients.

5.10   TRANSFORMATION OF P-2 INTO PLANTS

5

          It is also within the scope of this invention
that the P-2 gene (FIG. 2) be inserted directly into a
plant so that the plant itself produces the P-2 toxin.

10
          Genetic engineering of plants may be
accomplished by introducing the desired DNA containing
the P-2 gene into plant tissues or cells using DNA
molecules of a variety of forms and origins. These
include, but are not limited to: DNA molecules derived
from naturally occurring plant vectors such as the Ti
15
plasmid from Agrobacterium tumefaciens or plant pathogens
such as DNA viruses like Cauliflower Mosaic virus (CaMV)
or Geminiviruses, RNA viruses, and viroids; DNA molecules
derived from unstable plant genome components like
extrachromosomal DNA elements in organelles (e.g.,
20
chloroplasts or mitochondria), or nuclearly encoded
controlling elements; DNA molecules from stable plant
genome components (e.g., origins of replication and other
DNA sequences which allow introduced DNA to integrate
into the organellar or nuclear genomes and to replicate
25
normally, to autonomously replicate, to segregate
normally during cell division and sexual reproduction of
the plant and to be inherited in succeeding generations
of plants).

30
          DNA containing the P-2 gene may be delivered
into the plant cells or tissues directly by infectious
plasmids, such as Ti, viruses or microorganisms like A.
tumefaciens, the use of liposomes, microinjection by

35

mechanical methods and by whole chromosomes or chromosome
fragments.

5.11 PRODUCTS AND FORMULATIONS
5   INCORPORATING THE P-2 PROTEIN

        The P-2 delta-endotoxin is a potent
insecticidal compound with activity against lepidopteran
and dipteran insects. It is, therefore, within the scope
10 of the invention that the P-2 protein toxin be utilized
as an insecticide (the active ingredient) alone,
preferably in homogenous or pure form and having the
amino acid sequence of FIG. 2, or as included within or
in association with a transformed microorganism which
15 expresses a cloned P-2 gene or in a mixture of B.t. or
other transformed sporulating microorganisms containing
P-2 in spores or otherwise. The compositions of the
invention containing P-2 are applied at an insecticidally
effective amount which will vary depending on such
20 factors as, for example, the specific lepidopteran or
dipteran insects to be controlled, the specific plant to
be treated and the method of applying the insecticidally
active compositions. The preferred insecticide
formulations are made by mixing P-2 alone or incorporated
        in or associated with a transformed organism, with the
25 desired carrier. The formulations may be administered as
a dust or as a suspension in oil (vegetable or mineral)
or water, a wettable powder or in any other material
suitable for agricultural application, using the
appropriate carrier adjuvants. Suitable carriers can be
30 solid or liquid and correspond to the substances
ordinarily employed in formulation technology, e.g.,
natural or regenerated mineral substances, solvents,
dispersants, wetting agents, tackifiers, binders or
fertilizers.
35

The formulations containing a solid or liquid
adjuvant, are prepared in known manner, e.g., by
homogenously mixing and/or grinding the active
ingredients with extenders, e.g., solvents, solid
5   carriers, and in some cases surface active compounds
(surfactants).

Suitable liquid carriers are vegetable oils,
such as coconut oil or soybean oil, mineral oils or
10   water. The solid carriers used, e.g., for dusts and
dispersible powders, are normally natural mineral fibers
such as calcite, talcum, kaolin, or attapulgite. In
order to improve the physical properties it is also
possible to add highly dispersed silicic acid or highly
15   dispersed absorbent polymers. Suitable granulated
adsorptive carriers are porous types, for example pumice,
broken brick, seplolite or bentonite. Suitable
nonsorbent carriers are materials such as silicate or
sand. In addition, a great number of pregranulated
20   materials or inorganic or organic mixtures can be used,
e.g., especially dolomite or pulverized plant residues.

Depending on the nature of the active
ingredients to be formulated, suitable surface-active
compounds are non-ionic, cationic and/or anionic
25   surfactants having good emulsifying, dispersing and
wetting properties. The term "surfactants" will also be
understood as comprising mixtures or surfactants.

Suitable anionic surfactants can be both
30   water-soluble soaps and water-soluble synthetic surface
active compounds.

Suitable soaps are the alkali metal salts,
alkaline earth metal salts or unsubstituted ammonium
35

salts of higher fatty acids (C_{10}-C_{11}), e.g., the sodium
or potassium salts of oleic or stearic acid, or natural
fatty acid mixtures which can be obtained, e.g., from
coconut oil or tallow oil. Further stable surfactants
are also the fatty acid methyltaurin salts as well as
modified and unmodified phospholipids.

More frequently, however, so-called synthetic
surfactants are used, especially fatty sulfonates, fatty
sulfates, sulfonated benzimidazole derivatives or
alkylarylsulfonates.

The fatty sulfonates or sulfates are usually in
the forms of alkali metal salts, alkaline earth metal
salts or unsubstituted ammonium salts and generally
contain a C_6-C_{22} alkyl, e.g., the sodium or calcium salt
of dodecylsulfate, or of a mixture of fatty alcohol
sulfates, obtained from fatty acids. These compounds
also comprise the salts of sulfonic acid esters and
sulfonic acids of fatty alcohol/ethylene oxide adducts.
The sulfonated benzimidazole derivatives preferably
contain 2 sulfonic acid groups and one fatty acid radical
containing about 8 to 22 carbon atoms. Examples of
alkylarylsulfonates are the sodium, calcium or
triethanolamine salts of dodecylbenzenesulfonic acid,
dibutynaphthalenesulfonic acid, or of a
naphthalenesulfonic acid/formaldehyde condensation
product. Also suitable are corresponding phosphates,
e.g., salts of the phosphoric acid ester of an adduct of
p-nonylphenol with 4 to 14 moles of ethylene oxide.

Nonionic surfactants are preferbly polyglycol
ether derivative or aliphatic or cycloaliphatic alcohol
or saturated or unsturated fatty acids and alkylphenols,
said derivative containing 3 to 10 glycol ether groups

and 8 to 20 carbon atoms in the (aliphatic) hydrocarbon
moiety and 6 to 18 carbon atoms in the alkyl moiety of
the alkylphenols.

5   Other suitable non-ionic surfactants are the
water soluble adducts of polyethylene oxide with
alkypropylene glycol, ethylenediaminopolypropylene glycol
and alkylpolypropylene glycol contain 1 to 10 carbon
atoms in the alkyl chain, which aducts contain 20 to 250
10   ethylene glycol ether groups and 10 to 100 propylene
glycol ether groups.

          Representative examples of non-ionic
surfactants are nonylphenolpolyethoxyethanols, castor
15   oil, glycol ethers, polypropylene/polyethylene oxide
adducts, tributylphenoxypolyethoxyethanol, ethylene
glycol and octylphenoxypolyethoxynethanol. Fatty acid
esters of polyoxyethylene sorbitan, such as
polyoxyethylene sorbitan trioleate, are also suitable
20   non-ionic surfactants.

          Cationic surfactants are preferably quaternary
ammonium salts which contain, as substituents on the
nitrogen, at least one C_8-C_{22} alkyl radical and, as
25   further substituents, lower unsubstituted or halogenated
alkyl benzyl, or hydroxylated lower alkyl radicals. The
salts are preferably in the form of halides, methyl
sulfates or ethylsulfates, e.g., stearyltrimethylammonium
chloride.

30
5.12 CONCENSUS DELTA-ENDOTOXIN PROTEIN HOMOLOGY

          A computer search was conducted to determine
whether the P2 gene was homologous to any other genes
whose sequences had been published. No DNA sequence
35

homology was found between the P2 gene and other genes.
However, surprisingly a degree of amino acid sequence
homology was found to exist between the P2 and P1
proteins. Figure 6 shows that the P2 and P1 proteins

5   shared a region of 37% homology over a stretch of about
100 amino acids.

Sequences of conserved amino acids that are
found within otherwise non-homologous proteins often

10   signal important functional domains for the proteins. It
is forseeable that the conserved amino acids shown in
Figure 6 constitute an "active site" that is responsible
for the lepidopteran larvicidal activities of the P1 and
P2 proteins. Therefore, this protein has utility in site

15   specific mutagenesis experiments to effect or change the
amino acid composition so as to more specifically target
a resultant change in toxicity.

The importance of this stretch of 100 amino

20   acids can be determined by subcloning the 300-bp fragment
of DNA that encodes these amino acids. This procedure
would also yield a means for producing this protein in
B.t., B.megaterium or E. coli. Subcloning may be
accomplished by using site-specific in vitro mutagenesis

25   to create restriction sites bordering the 300-bp fragment
of DNA coding for this one-hundred amino acid protein.
These restriction sites could then be used to precisely
excise the DNA fragment. The DNA fragment could then be
inserted downstream from a promoter and ribosome binding

30   site on a Bacillus or other appropriate vector. The
Bacillus vector could be genetically engineered in such a
way that it would contain the promoter and ribosome
binding site of the P2 gene itself. The resulting
recombinant plasmid could then be transformed into an

35   appropriate Bacillus strain (or other appropriate

organism as described herein) and the larvicidal activity
of the recombinant strain could be measured. The
transformed organism would also serve as a means for
producing this protein. The transformed organism or the
5   protein itself or both in admixture may be utilized in an
insecticidal composition in the same manner as the P2
toxin protein.

It is possible that the 100 amino acid
10   polypeptide that would be synthesized from the
recombinant plasmid would be degraded by proteases within
the Bacillus cell. To circumvent this potential problem
a protease negative strain of Bacillus could be used for
expression such as the one described by Wong et al.
15   (Wong, S., Kawamura, F., and Doi, R. 1986 J. Bacteriol.
168:1005-1009).

6.0   EXAMPLES

The insecticidal activity of transformed or
20   non-transformed Bacillus megaterium and of Escherichia
coli was determined by including various amounts of these
microorganisms in a test diet which was fed to insects.
After feeding, insect mortality was measured.
Specifically, this involved growing the microorganism to
25   stationary phase on solid agar Base media for two days at
30°C. For E. coli harboring plasmids the media was LB
containing 40 ug/ml ampicillin. For B. megaterium
harboring plasmids the media was DS containing 10 ug/ml
tetracycline. The microorganisms were harvested from the
30   solid medium by scraping with a spatula. The wet weight
of the harvested bacteria was determined and bacterial
cells were resuspended to a known concentration in
deionized water. Serial dilutions of the suspended
35   bacterial cells were made and 200 ul of each dilution was

topically applied to 3 ml of a solid agar-based
artificial diet in a feeding cup. The top surface area
of the diet was 600 square millimeters. In the case of
Heliothis the diet contained soy flour and for Lymantria
5   dispar the diet contained wheat germ. One neonate larvae
was placed in each feed cup and mortality was scored
after seven days.

The LC50 value (weight of bacterial cells
10 required to kill 50% of the larvae) was calculated from a
probit analysis of insect mortality (R.J. Daum., A
Revision of Two Computer Programs for Probit Analysis.
Bulletin of the Entomological Soc. of America, vol 16(1),
pp. 1-15).

15

6.1   EXAMPLE 1 - BIOASSAY OF THE EXPRESSION
      PRODUCT OF THE CLONED P-2 GENE IN E. COLI

Heliothis virescens larvae were fed a standard
diet to which had been added E. coli cells harboring
20 various plasmids and known to either have or not have the
P-2 gene present. After seven days on the diet the
larvae were scored for growth and viability with the
results reported below in Table I. It is apparent from
these results that the cloned P-2 gene is, in fact,
25 expressed in E. coli, a non-sporulating bacteria, and
that the product of the expression of this cloned gene
renders the transformed E. coli significantly more toxic
to Heliothis virescens larva than E. coli without the P-2
gene present.

30

35

TABLE I

E. coli (plus Plasmid)	Dose	H. virescens larvae dead or stunted/total
Strain EG1303 (pBR322 no P-2 gene)	10 mg/cup	2/20
Stain EG1304 (pEG201 P-2 gene present)	10/mg/cup	20/20

6.2 **EXAMPLE 2 - TRANSFORMATION OF THE**
     P-2 GENE INTO BACILLUS MEGATERIUM

Plasmid pEG201 (containing the P-2 gene) will
replicate only in gram-negative strains such as E. coli.
The P-2 gene was expressed in E. coli strain EG1304
harboring pEG201 but only at a low level (see Bioassay
data, Table I). The purpose of this example was to
determine whether the cloned P-2 gene would be expressed
at a higher level in other Bacillus strains. In order to
test for the expression of the cloned P-2 gene in a
Bacillus strain it was first necessary to construct a
recombinant plasmid that contained the P-2 gene and that
was capable of replicating in Bacillus. A Bacillus-E.
coli "shuttle vector" that contained the P-2 gene was
constructed. The term "shuttle vector" indicates that
the plasmid is capable of replication both in Bacillus
and in E. coli. The E. coli - Bacillus shuttle vector

was constructed by digestion of the Bacillus plasmid
pBC16 (tetracycline resistance) with SphI, ligation of
the digested plasmid into the SphI site of pEG201
(ampicillin resistance) and transformation of E. coli to
ampicillin and tetracycline resistance.

One tet and amp resistant E. coli transformant
harbored a plasmid (designated pEG204) that was composed
of pBC16 inserted into the SphI site of pEG201 (Figure
1). Figure 1 shows the restriction map of plasmid
pEG204. The boxed areas denote plasmid vector DNA. The
open box is pBR322 DNA (E. coli replication) and the
cross-hatched box is pBC16 DNA (Bacillus replication).
The horizontal line is cloned DNA from strain HD263-1.
The large arrow denotes the coding region of the P-2
gene. pEG204 was transformed into Bacillus megaterium
(ATCC deposit number VT 1660)and one tetracycline
resistant transformant harboring pEG204 (designated
strain EG1312) was chosen for further study.

This example determined if the cloned P-2 gene
was expressed in the recombinant B. megaterium strain
EG1312 (pEG204). Gene expression was measured by the
technique of NadodeSO4/polyacrylamide gel
electrophoresis. Generally, the technique involved
preparation of cell lysates, electrophoresis of cell
lysates through a NadodeSO4/polyacrylamide gel and
staining of the gel to permit visualization of proteins.

Specifically, the technique was carried out as
follows: B. megaterium cells were grown on DS plates
containing 10ug/ml tetracycline for 48 hr. at 30°C. B.
thuringiensis strains were grown similarly to B.
megaterium except the DS plates contained no
tetracycline. After this period almost all cells had

entered the stationary phase of growth. Cells were
harvested with a spatula and resuspended in deionized
water. A portion of the cell suspension was mixed 1:2
vol:vol with preheated (70°C) gel loading buffer (5% Beta
-mercaptoethanol, 2% NaDodeSO4, 60 mM Tris pH 6.8, 10%
glycerol) and incubated at 70°C for 7 min. The
suspension was centrifuged briefly, after centrifugation
the supernatant was immediately loaded onto an
NadodeSO4/polyacrylamide gel and the proteins in the
supernatant were resolved by gel electrophoresis
according to the method of Laemmli. (J. of Mol. Bio.,
80:576-599 (1973)) The proteins in the gel were
visualized by staining the gel with Coomassie dye.

        Figure 5 shows the results of this analysis.
Figure 5 is a photograph of an NadodeSO4/polyacrylamide
gel that had been prepared as described above. The lane
labeled STND in Fig. 5 contained protein molecular weight
standards. Numbers to the right of the gel indicate
protein sizes in kilodaltons (kDa). The lane labeled
HD1-1 contained extracts of that B.t. strain. The major
protein band that corresponded to P-2 protein is
indicated by an arrow. The lane labeled P2 contained a
portion of the purified P-2 protein. The P2 protein was
purified as described above.

        The lanes labeled EG1311 and EG1312 in Fig. 5
contained extracts of these B. megaterium strains
harboring pBC16 and pEG204(P2) respectively. A
comparison of lanes EG1311 and EG1312 showed that
extracts of strain EG1312(pEG204) contained a major
protein that corresponded in size to that of the P2
protein. This protein was not present in extracts of
strain EG1311(pBC16). This demonstrates that B.
megaterium harboring the cloned P-2 gene synthesized high

-42-

levels of P2 protein. In addition, when viewed under the
light microscope the cells of strain EG1312 appeared to
contain phase-bright protein inclusion bodies
characteristic of crystal toxins.

5

10

15

20

25

30

35

-43-

6.3   BIOASSAY OF THE EXPRESSION PRODUCT OF
        THE CLONED P-2 GENE IN B. MEGATERIUM

Standard toxicity tests carried out indicated
5   that strain Bacillus megaterium EG1312 had an LD50 (50%
of larvae dead) of 1.4 ug of bacterial cells per insect
food cup when fed to either Heliothis virescens (H.v.) or
to Lymantria dispar (L.d.) larvae. In contrast the
control strain of Bacillus megaterium harboring the
10 plasmid vector pBC16 without the P-2 gene failed to kill
either H.v. or L.d. larvae at a dose of 10 ug bacterial
cells per food cup.

B. megaterium strain EG1312 (pEG204-P2) was
15 also tested for toxicity against A. aegypti. A cell
suspension was prepared by growing strains EG1311(pBC16-
negative control) and EG1312 on solid DS medium ·
containing 10 ug/ml tetracycline for 48 hr. at 30°C.
Cells were harvested with a spatula and cells were
resuspended in deionized water. Serial dilutions of the
20 cell suspensions were made. Twenty larvae (third or
fourth instar) of A. aegypti were placed in 100ml of the
cell suspensions and mortality was scored after 48 hr.
The results (below) showed that strain EG1312(P2) is
toxic to mosquito larvae. In contrast B. megaterium
25 containing the vector plasmid PBC16 alone (strain EG1311)
was not toxic to mosquito larvae.

30

35

TABLE II

5   Dose-mg cells/ml

	A. aegypti larvae # dead/total
EG1311(pBC16 control)-0.8mg/ml	0/20
EG1312(pEG204-P2)-0.8mg/ml	10/20
-0.4mg/ml	12/20
-0.2mg/ml	10/20
-0.1mg/ml	9/20
EG1311(control)-0.9mg/ml	0/20
EG1312(pEG204-P2)-0.9mg/ml	16/20
-0.8mg/ml	16/20
-0.4mg/ml	15/20
-0.2mg/ml	12/20
-0.1mg/ml	12/20

25   7.0   DEPOSIT OF MICROORGANISMS

It is within the scope of this invention that a
wide variety of both sporulating and nonsporulating
microorganisms may be transformed with the P-2 as
described herein. Exemplary of the microorganisms which
may be engineered as taught herein are those from the
genera _Bacillus_, _Escherichia_, and _Cyanobacteria_.
Preferred for use with this invention are the organisms
Bacillus megaterium and _Escherichia coli_. In addition,
the following _Bacillus thuringiensis_, _Bacillus megaterium_

and E. coli strains which are also preferred for use
with this invention and which carrying the listed
plasmids have been deposited with the Agricultural
Research Culture Collection (NRRL), Peoria, IL and have
been assigned the listed accession numbers:

B. thuringiensis stain	Plasmids	Accession Numbers
HD1-1	Several naturally occuring	B-18201
HD263-1	"	B-18202

B. megaterium	Plasmid	Accession Numbers
EG1312	pEG204	B-18203

E. coli	Plasmid	Accession Numbers
EG1304	pEG201	B-18204

The present invention is not to be limited in
scope by the microorganism deposited, since the deposited
embodiment is intended as a single illustration of one
aspect of the invention. Indeed, various modifications
of the invention in addition to those shown and described
herein will become apparent to those skilled in the art
from the foregoing description and accompanying drawings.

I claim:

1.   A gene for <u>Bacillus</u> <u>thuringiensis</u> P-2 toxin having
the DNA sequence of FIGURE 2 or any portions or
derivatives thereof.

2.   The gene of claim 1 wherein said gene codes for a
protein having the amino acid sequence of FIGURE 2.

3.   The gene of claim 2 wherein said protein has
insecticidal activity.

4.   The gene of claim 3 wherein said insecticidal
activity is effective against insects selected from the
families consisting of lepidoptera and diptera.

5.   The gene of claim 1 wherein said DNA sequence is
inserted into a recombinant plasmid.

6.   The gene of claim 5 wherein said plasmid is comprised
of DNA from at least two different species of
microorganisms after insertion of said DNA sequence.

7.   The gene of claim 5 wherein said plasmid is comprised
of DNA from at least two different subspecies of the same
species of microorganism after insertion of said DNA
sequence.

8.   The gene of claim 1 wherein said DNA sequence is
attached to its native promoter DNA sequence.

9.   The gene of claim 1 wherein said DNA sequence is
attached to a foreign promoter DNA sequence.

10.   A protein having the amino acid sequence of FIGURE 2 or any portions or derivatives thereof.

11.   The protein of claim 10 wherein said protein has
5   insecticidal activity.

12.   The protein of claim 11 wherein said insecticidal activity is effective against insects selected from the families consisting of lepidoptera and diptera.

10

13.   The protein of claim 10 wherein said protein is produced by the process comprising:

        a)   transforming a microorganism with the gene
15   of FIGURE 2;

        b)   growing said transformed microorganism whereby the protein encoded by said gene of step a) is expressed in said microorganism and

20

        c)   extracting and separating said protein expressed in step b) from said organism.

14.   The protein of claim 13 wherein said gene of step a)
25   is located on a plasmid.

15.   The protein of claim 14 wherein said plasmid is comprised of DNA from at least two different species of microorganisms when including said gene.

30

16.   The protein of claim 14 wherein said plasmid is comprised of DNA from at least two different subspecies of microorganism when including said gene.

35

17. The protein of claim 13 wherein said protein is expressed in a non-sporulating microorganism.

18. The protein of claim 15 and 16 wherein the gene is controlled by its native promoter.

19. The protein of claim 15 and 16 wherein the gene is controlled by a foreign promoter.

20. The protein of claim 19 wherein said protein is expressed in a sporulating microorganism.

21. The protein of claim 18 wherein the protein is expressed during non-sporulating growth phases of said microorganism.

22. The protein of claim 13 wherein said protein is extracted in step c) by lysis of said microorganism.

23. The protein of claim 10 wherein said protein is in substantially pure form.

24. A method for producing of Bacillus thuringiensis P-2 toxin comprising:

    a)   inserting into a plasmid a gene for said P-2 toxin having the DNA sequence of Figure 2;

    b)   transforming a microorganism with the plasmid of step a) and

-49-

     c)   growing the transformed microorganisms
          of step b) whereby said P-2 toxin is
          expressed in said microorganisms.

 5   25. The method of claim 24 wherein said gene codes for a
     protein having the amino acid sequence of FIGURE 2.

     26. The gene of claim 24 wherein said plasmid is
     comprised of DNA of at least two different species of
10   microorganism after insertion of said P-2 gene.

     27. The method of claim 24 wherein said plasmid is
     comprised of DNA from at least two different subspecies
     of the same species of microorganism after insertion of
15   said P-2 gene.

     28. The method of claim 24 wherein said DNA sequence is
     attached to its native promoter DNA sequence.

     29. The gene of claim 24 wherein said DNA sequence is
20   attached to a foreign promoter DNA sequence.

     30. The method of claim 24 wherein said microorganism is
     a non-sporulating microorganism.

25
     31. The method of claim 21 wherein said microorganism is
     a sporulating microorganism.

     32. The method of claim 30 wherein the P-2 toxin is
     expressed during non-sporulating growth phases of said
30   microorganism.

     33. The method of claim 24 wherein said P-2 toxin is
     extracted from the microorganism by lysis of said
     microorganism.
35

-50-

34. An insecticide suitable for use against Lepidoptera
comprising a mixture of a Bacillus thuringiensis P-2
toxin and a suitable carrier.

5   35.   The insecticide of claim 34 wherein the P-2 toxin
is associated with Bacillus thuringiensis spores.

36.   The insecticide of claim 34 wherein the P-2 toxin is
a homogeneous protein preparation.

10

37.   The insecticide of claim 34 wherein the P-2 toxin is
contained in a mixture of Bacillus thuringiensis spores
and cultured Bacillus thuringiensis organisms.

15   38.   The insecticide of claim 34 wherein the P-2 toxin is
associated with a non-sporulating microorganism.

39.   The insecticide of claim 34 wherein the P-2 toxin is
associated with a sporulating microorgamism.

20
40.   The insecticide of claim 34 wherein the carrier is a
liquid carrier.   .

41.   The insecticide of claim 40 wherein the liquid
carrier contains one of more surfactants.
25

42.   The insecticide of claim 34 wherein the carrier is a
solid carrier.

30   43.   The insecticide of claim 43 wherein the solid
carrier is selected from the group consisting of calcite,
talcum, koalin, attapulgite, silicate, sand, dolomite,
and pulverized plant residue.

35

-51-

44. The insecticide of claim 43 wherein the solid
carrier is a granulated adsorptive carrier.

45. The insecticide of claim 45 wherein the granulated
adsorptive carrier is selected from the group consisting
of pumic, broken brick, seplolite, and bentonite.

46. An insecticide suitable for use against *Diptera*
comprising a mixture of a *Baccilus* *thuringiensis* P-2
toxin.

47. The insecticide of claim 46 wherein the P-2 toxin is
associated with a non-sporulating microorganims.

48. The insecticide of claim 46 wherein the P-2 toxin is
associated with a sporulating mircoorganism.

49. The insecticide of claim 46 wherein the carrier is a
liquid carrier.

50. The insecticide of claim 49 wherein the liquid
carrier is selected from the group consisting of water,
vegetble oil, and mineral oil.

51. The insecticide of claim 49 wherein the liquid
carrier contains one or more surfactants.

52. The insecticide of claim 46 wherein the carrier is a
solid carrier.

53. The insecticide of claim 52 wherein the solid
carrier is selected from the group consisting of calcite,
talcum, kaolin, attapulgite, silicate, sand, dolomite,
and pulverized plant residue.

-52-

54.   The insecticide of claim 52 wherein the solid carrier is a granulated adsorptive carrier.

55.   The insecticide of claim 5̶5̶ [54] wherein the granulated WFD adsorptive carrier is selected from the group consisting 4/16/8 of pumice, broken brick, seplolite, and bentonite.

56.   The insecticide of claim 46 wherein the P-2 toxin is associated with Bacillus thuringiensis spores.

57.   The insecticide of claim 46 wherein the P-2 toxin is a homogenous protein preparation.

58.   The insecticide of claim 46 wherein the P-2 toxin is contained in a mixture of Bacillus thuringiensis spores and non-sporulated Bacillus thuringiensis organisms.

59.   A recombinant vector containing the DNA sequence of claim 1.

60.   A non-sporulating microorganism containing the DNA sequence of claim 1.

61.   The non-sporulating microorgnism of claim 60 wherein said microorganism is E. coli.

62.   A sporulating microorganism containing the DNA sequence of claim 1.

63.   A microorganism containing the DNA sequence of claim 1 selected from the group consisting of Bacillus, Escherichia and Cyanobacteria.

-53-

64. A _Escherichia_ _coli_ bacterium deposited with NRRL
and assigned Accession No B-18204, or a mutant,
recombinant, or genetically engineered derivative
thereof.

5

65. A _Bacillus_ _megaterium_ bacterium deposited with NRRL
and assigned Accession No. B-18203, or a mutant,
recombinant, or genetically engineered derivative
thereof.

10

66. An oligonucleotide probe for the gene coding for P-2
delta-endotoxin comprising the sequence:

> 5'GTA TTA AAT TCA GGA AGA ACA ACA AAT AAT GAT
> GCA TAT AAT GTA GTA GCA CAT GAT CCA TT-3',

15

or derivative thereof.

67. The oligonucleotide probe of claim 66 wherein said
probe is labeled.

20

68. The oligonucleotide probe of claim 67 wherein said
probe is labeled with a radioactive label.

69. The DNA sequence of FIGURE 2 wherein said DNA or a
portion or derivative thereof is labeled.

25

70. The DNA sequence of Claim 69 wherein said DNA or
portion or derivative thereof is labeled with a
radioactive label.

30

71. A plant transformed with the DNA sequence of FIGURE
2.

35

-54-

72. The plant of claim 71 wherein the plant produces P-2 toxin.

73. A protein having the amino acid sequence of a
5 fragment ~~of a fragment~~ of P-2 as in FIGURE. 6.   *WFO* 4/16/87

74. A protein having the amino acid sequence of a fragment of P-1 as indicated in FIGURE. 6.

10 75. A protein having about 100 amino acids wherein the amino acids comprise in composition and sequence at least those which are indicated as being shared between P-1 and P-2 in FIGURE 6.
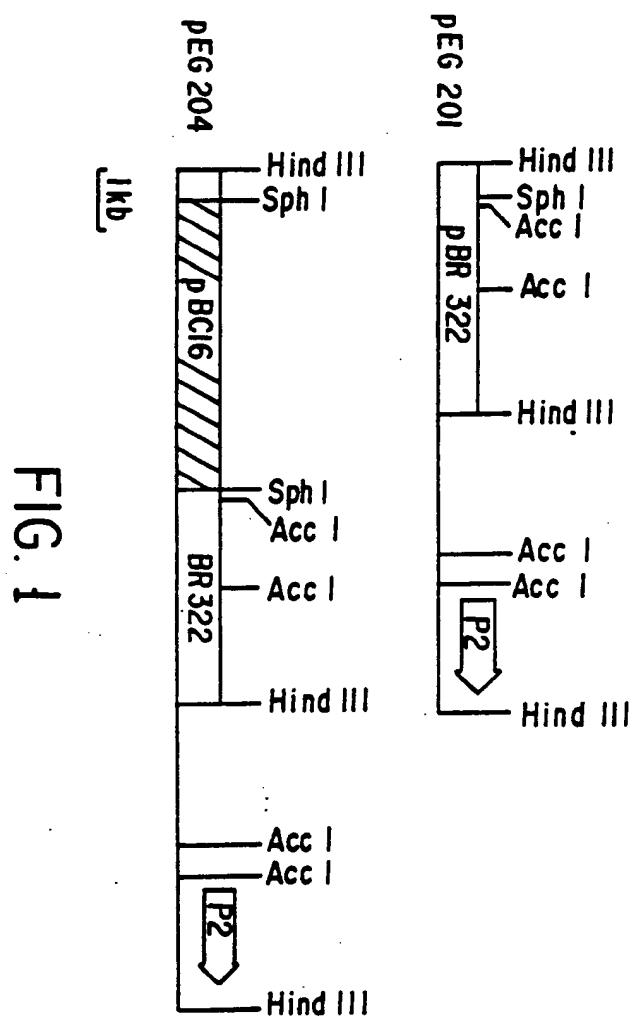
15

20

25

30

35

FIG. 1

Acc I   10   20   30   40   50   60   F I G .
GTATACACACAAGATTTAATTGATACGTATAATCAAAGTCAGAATTGTGATTGTGGTTGT

                                                                                    ~/~
         70   80   90   100   110   120
AAGTAGTAAGTAGTAAGTAGTTTCTTAAACATACTCGTTATTATCAAAAGAGTTTAGTTT

         130   140   150   160   170   180
TAATATAAAACTAGATATTTAAGGAGGAATTTTATATGAATAATGTATTGAATAGTGGAA
                                            MetAsnAsnValLeuAsnSerGlyA

         190   200   210   220   230   240
GAACAACTATTTGTGATGCGTATAATGTAGTAGCCCATGATCCATTTAGTTTTGAACATA
rgThrThrIleCysAspAlaTyrAsnValValAlaHisAspProPheSerPheGluHisL

         250   260   270   280   290   300
AATCATTAGATACCATCCAAAAAGAATGGATGGAGTGGAAAAGAACAGATCATAGTTTAT
ysSerLeuAspThrIleGlnLysGluTrpMetGluTrpLysArgThrAspHisSerLeuT

         310   320   330   340   350   360
ATGTAGCTCCTGTAGTCGGAACTGTGTCTAGTTTTTTGCTAAAGAAAGTGGGGAGTCTTA
yrValAlaProValValGlyThrValSerSerPheLeuLeuLysLysValGlySerLeuI

         370   380   390   400   410   420
TTGGAAAAAGGATATTGAGTGAATTATGGGGGATAATATTTCCTAGTGGTAGTACAAATC
leGlyLysArgIleLeuSerGluLeuTrpGlyIleIlePheProSerGlySerThrAsnL

         430   440   450   460   470   480
TAATGCAAGATATTTTAAGGGAGACAGAACAATTCCTAAATCAAAGACTTAATACAGATA
euMetGlnAspIleLeuArgGluThrGluGlnPheLeuAsnGlnArgLeuAsnThrAspT

         490   500   510   520   530   540
CCCTTGCTCGTGTAAATGCAGAATTGATAGGGCTCCAAGCGAATATAAGGGAGTTTAATC
hrLeuAlaArgValAsnAlaGluLeuIleGlyLeuGlnAlaAsnIleArgGluPheAsnG

         550   560   570   580   590   600
AACAAGTAGATAATTTTTTAAACCCTACTCAAAACCCTGTTCCTTTATCAATAACTTCTT
lnGlnValAspAsnPheLeuAsnProThrGlnAsnProValProLeuSerIleThrSerS

         610   620   630   640   650   660
CGGTTAATACAATGCAGCAATTATTTCTAAATAGATTACCCCAGTTCCAGATACAAGGAT
erValAsnThrMetGlnGlnLeuPheLeuAsnArgLeuProGlnPheGlnIleGlnGlyT

         670   680   690   700   710   720
ACCAGTTGTTATTATTACCTTTATTTGCACAGGCAGCCAATATGCATCTTTCTTTTATTA
yrGlnLeuLeuLeuLeuProLeuPheAlaGlnAlaAlaAsnMetHisLeuSerPheIleA

         730   740   750   760   770   780
GAGATGTTATTCTTAATGCAGATGAATGGGGTATTTCAGCAGCAACATTACGTACGTATC
rgAspValIleLeuAsnAlaAspGluTrpGlyIleSerAlaAlaThrLeuArgThrTyrA

         790   800   810   820   830   840
GAGATTACCTGAGAAATTATACAAGAGATTATTCTAATTATTGTATAAATACGTATCAAA
rgAspTyrLeuArgAsnTyrThrArgAspTyrSerAsnTyrCysIleAsnThrTyrGlnT

         850   860   870   880   890   900
CTGCGTTTAGAGGGTTAAACACCCGTTTACACGATATGTTAGAATTTAGAACATATATGT
hrAlaPheArgGlyLeuAsnThrArgLeuHisAspMetLeuGluPheArgThrTyrMetP

```
         1810        1820        1830        1840        1850        1860
TATCTTCAATAGGAAATTCAACTATTCGAGTTACTATAAACGGTAGAGTTTATACTGTTT
alSerSerIleGlyAsnSerThrIleArgValThrIleAsnGlyArgValTyrThrValS
```

```
         1870        1880        1890        1900        1910        1920
CAAATGTTAATACCACTACAAATAACGATGGAGTTAATGATAATGGAGCTCGTTTTTTCA
erAsnValAsnThrThrThrAsnAsnAspGlyValAsnAspAsnGlyAlaArgPhePheA
```

```
         1930        1940        1950        1960        1970        1980
GATATTAATATCGGTAATATAGTAGCAAGTGATAATACTAATGTAACGCTAGATATAAAT
rgTyr
```

```
         1990        2000        2010        2020        2030        2040
GTGACATTAAACTCCGGTACTCCATTTGATCTCATGAATATTATGTTTGTGCCAACTAAT
```

```
         2050        2080        2070        2080        2090        2100
CTTCCACCACTTTATTAAGGTTTGAGTGAATGTACAATTAGTATTTTATTCTATCATAAA
```

```
         2110        2120        2130        2140        2150        2160
TTTAATAGAAAATTCTTAAACATATTGACGGAACTAAATGATATATAATTATGGATATTA
```
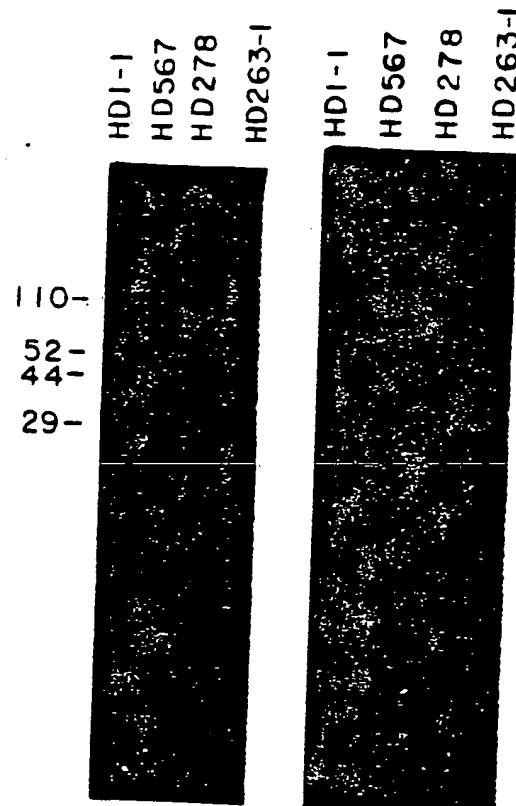
```
         2170        2180        2190        2200        2210        2220
GAGGGTGTCTTAAAGTAGTAAAATTCTTACTCTGAGACACCCTCTTTATTTTTTATATC
```

```
                                                         Hind III
         2230        2240        2250        2280        2270
CAAATCGGATGAAATATGGGAGAAATCATTTCAAGTTAACCTAAAAGCTT
```

FIG. 2

~3~

F I G. 3

3A   3B

F I G .   4

4A   4B   4C
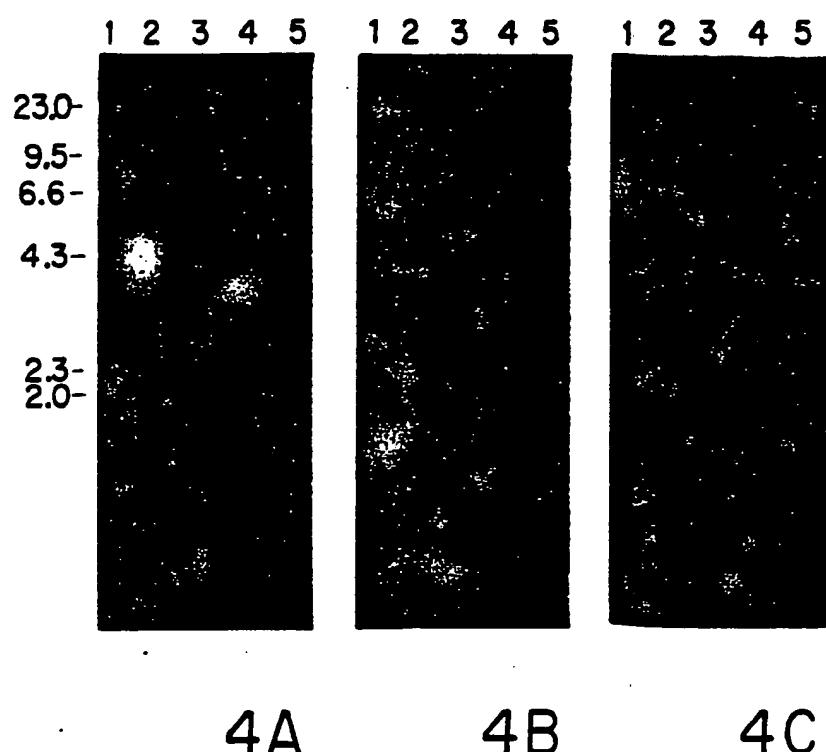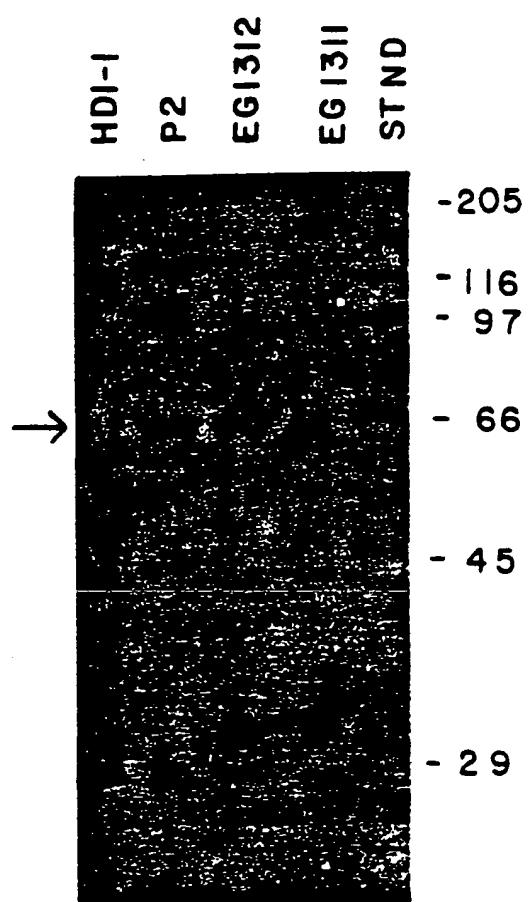
FIG. 5

FIG. 6

HOMOLOGY BETWEEN P 2
AND P 1 AMINO ACID SEQUENCES

P2 protein
Parasporal crystal protein - Bacillus thuringiensis (fragment)

Amino
Acid
Number

```
162   ProGlnPheGlnIleGlnGlyTyrGlnLeuLeuLeuLeuProLeuPheAlaGlnAlaAla       P2
146   ProLeuLeuAlaValGlnAsnTyrGlnValProLeuLeuSerValTyrValGlnAlaAla  ·    P1
          *    *  *  *          *        *  *  *      *  *  *

132   AsnMetHisLeuSerPheIleArgAspValIleLeuAsnAlaAspGluTrpGlyIleSer       P2
166   AsnLeuHisLeuSerValLeuArgAspValSerValPheGlyGlnArgTrpGlyPheAsp       P1
      *            *                                   *

2C2   AlaAlaThrLeuArgThr       TyrArgAspTyrLeuArg                       P2
186   AlaAlaThrIleAsnSerArgTyrAsnAspLeuThrArgLeuIleGlyAsnTyrThr         P1
          *      *                         *

218   AspTyrSerAsnTyrCysIleAsnThrTyrGlnThrAlaPheArgGlyLeuAsnThrArg       P2
205   AspTyrAlaValArgTrpTyrAsnThrGlyLeuGluArgValTrpGlyProAspSerArg       P1
      *  *          *            *      *        *

238   LeuHisAspMetLeuGlu    PheArgThrTyrMetPheLeuAsnValPheGluTyrVal       P2
225   AspTrpValArgTyrAsnGlnPheArgArgGluLeuThrLeuThrValLeuAspIleVal       P1
      *        *              *  *              *  *

257   SerIleTrpSer       260    P2
245   AlaLeuPheSer       248    P1
      *  *
```

Matches = 39　　　　　　Length = 104　　　　　　Matches/length = 37.5 percent

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/01132

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): C12P 21/00; C12N 1/20; C07K 13/00;C07H 15/12;A01H 1/04
U.S. CL.: 435/68,253; 530/350; 536/27; 800/1

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/68,253; 530/350; 536/27

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

DATABASE:Chemical Abstracts Services Online (File CA, 1967–1987;File Biosis, 1969-1987). Automated Patent System (File USPAT, 1975-1987). Bacillus thuringiensis,P-2,P2,P(w)2,inventor

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	GENE, Volume 36, issued 1985, (Amsterdam, the Netherlands), Aaang et al., "Characterized full-length and truncated plasmid clones of the crystal protein of Bacillus thuringiensis subsp. kurstaki HD-73 and their toxicity to Manduca sexta," pages 289-300, see pages 291-297 in particular.	1,59,73
Y	US, A, 4,467,036 (SCHNEPF ET AL.), 21 August 1984 (21.08.84), see entire document.	5,6,8, 9,24-30, 32,33, 59
Y	SCIENCE, Volume 219, issued February 1983, (Washington, D.C.) MILLER ET AL., "Bacterial, Viral and Fungal insecticides," pages 715-721, see page 720 in particular.	5-9,13- 33,59

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 JULY 1988	1 0 AUG 1988

International Searching Authority	Signature of Authorized Officer
ISA/US	CHARLES E. COHEN

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document. with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	B.D. HAMES ET AL., "Nucleic Acid Hybridisation A Practical Approach", published 1985, by IRL Press (Oxford), see page 30.	69,70
X/Y	Chemical Abstracts, Volume 96, No. 3, issued 18 January 1982 (Columbus, Ohio, U.S.A.), T. YAMAMOTO ET AL., "Isolation of a protein from the parasporal crystal of <u>Bacillus thuringiensis</u> var. <u>kurstaki</u> toxic to the mosquito larva, <u>Aedes taeniorhynchus</u>," see page 196, column 2, the abstract No. 17020m, Biochem. Biophys. Res. Commun. 1981. 103(2), 414-21 (Eng.) (Section 10).	10-23, 73 ⎯⎯ 31, 33-58
X/Y	Chemical Abstracts, Volume 99, No. 17, issued 24 October 1983 (Columbus, Ohio, USA), T. YAMAMOTO, "Identification of entomocidal toxins of Bacillus thuringiensis by high performance liquid chromatography", see page 316, column 2, the abstract No. 136525b, J. Gen. Microbiol. 1983 129(8), 2595-603 (Eng.) (Section 10)	10-23, 73 ⎯⎯ 31, 33-58
X/Y	Chemical Abstracts, Volume 99, No. 19, issued 07 November 1983, (Columbus, Ohio, USA), T.IIZUKA ET AL. "Possible location of the mosquitocidal protein in the crystal preparation of Bacillus thuringiensis subsp. kurstaki," see page 316, column 2, the abstract No. 154890w (FEMS Microbiol. Lett. 1983. 19(2-3), 187-92 (Eng.) (Section 10)	10-23, 73 ⎯⎯ 31, 33-58
Y,P	US, A, 4,695,455 (BARNES ET AL.) 22 September 1987 (22.09.87), see entire document.	5-9, 13-33, 59, 34-58

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____ , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____ , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers _____ , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

   See attachment.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

   1-59, 69 ,70 and 73   Telephone Practice.

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

   ☐ The additional search fees were accompanied by applicant's protest.

   ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 11-87)

Attachment to Form PCT/ISA/210,
Part VI.

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING:

Group I: Claims 1-9, 26, 29, 59, 69 and 70, drawn to a
            gene for B. thuringiensis P-2 toxin; class
            536/27.

Group II: Claims 10-25, 27, 28, 30-58 and 73, drawn to
            P-2 toxin protein, a method for producing P-2
            toxin, and an insecticide; class 530/350,
            class 435/68, and class 435/253.

Group III: Claims 60-65, drawn to bacterial cells; class
            435/253.

Group IV: Claims 66-68, drawn to an oligonucleotide pro-
            probe; class 536/27.

Group V: Claims 71 and 72, drawn to a transformed
            plant; class 800/1.

Group VI: Claim 74, drawn to a P-1 protein fragment;
            class 530/350

Group VII: Claim 75, drawn to a P-1/P-2 hybrid-type pro-
            tein; class 530/350.